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Bovine Leukosis: Transmission

R. F. CROSS

OHIO AGRICULTURAL RESEARCH AND DEVELOPMENT CENTER
Wooster, Ohio

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BOVINE LEUKOSIS: TRANSMISSION

R. F. CROSS

INTRODUCTION

The purpose of this bulletin is to bring together the information obtained during a study of bovine malignant lymphoma. Information previously published has been briefly summarized when necessary. Previously unpublished material is presented in more detail.

Some negative results are unavoidable. Where these are presented, an attempt has been made to arrange them so that the interested reader can get enough detail to avoid repeating the process and the uninterested reader can easily skip over them.

Numerous people were involved in the study at various times and it was necessary for someone to organize a variety of observations into one permanent record. The author had the records at the time the study was terminated. His position is that of reporter. The observations were made by many.

CATTLE TRANSMISSION TRIALS

Materials and Methods

The Ohio Agricultural Research and Development Center (OARDC) has a dairy herd which varies in size from year to year but usually has between 225 and 250 cattle. Approximately two-thirds of these cattle are Holstein-Friesians and the rest are Jerseys.

The initial case of malignant lymphoma occurred in a 6-year-old Holstein-Friesian. This animal had rapidly enlarging, multiple lymphomatous tumors of the reticulum cell type. It became moribund and was euthanized after a very short course of 11 days.

Aseptically collected samples of the tumor masses were used to prepare a 10% cell suspension. Ten ml. of this suspension were given intravenously to each of four 6-month-old dairy heifers. Additional samples of tumor material were used to make a 10% suspension which was then filtered to remove all particulate matter of bacterial size or larger. Four additional 6-month-old dairy heifers were each given 10 ml. of this cell-free filtrate intravenously. The inoculated heifers had been born and housed in the facilities of the OARDC dairy. No special preinoculation preparations or postinoculation separations were made.

At 18 months postinoculation, one of the heifers given the cell-free filtrate developed malignant lymphoma of the same type and died after a course of 10 days. The details of this trial have been reported (11).

Because of the similarity in clinical course and postmortem lesions between the naturally occurring case and the inoculated heifer, a filtrate was made with tumor samples collected from the inoculated heifer. It was given intravenously to each of three calves. Portions of the filtrate from the original case which had been stored at —25°C. were given to two additional calves. Five bovine fetuses were inoculated *in utero* with filtrate from both the original case and the possible transmission.

In cooperation with the meat inspection division of the Cleveland Board of Health and practicing veterinarians in the Wooster area, additional cases of bovine malignant lymphoma were identified and samples of fresh tumor material were obtained. Additional calves were inoculated with leukemic blood, tumor cell suspensions, cell-free filtrates of tumors, or a DNA-RNA extract. These animals were maintained in an isolated unit under veterinary supervision and hemograms were made at monthly intervals. Four uninoculated animals were maintained in this unit as contact controls. Additionally, three calves born in the inoculated group and three calves born in the control group were maintained as contact controls. The details of inoculation are given in Table 1.

The Bendixen leukosis key (9) was used as a means of measuring lymphocytosis. An animal was considered to have persistent lymphocytosis if more than half of the hemograms were in the Bendixen positive range after the initial Bendixen positive reaction.

All animals were under 6 months of age when inoculated. They were killed and examined at ages ranging from 30 to 69 months. The average age at death was 40 months. Samples of the internal organs and lymph nodes of each animal were collected for microscopic examination.

Results

The results of the initial transmission trial have been reported (11). Neither gross nor microscopic evidences of malignant lymphoma were found in any animal inoculated after that time. No lesions were found in any control animal. Two inoculated animals (25 and 1735) developed persistent lymphocytosis but both of these animals were naturally infected with *Trypanosoma theileri*. Investigations made during this project showed that *T. theileri* could cause lymphocytosis in the Bendixen positive range and that approximately 1 in every 5 Bendixen positive samples contained demonstrable trypanosomes, while only 1 in every

20 Bendixen negative samples had demonstrated trypanosomes.

The results are summarized in Table 2.

HERD SURVEY

Materials and Methods

To find the prevalence of lymphocytosis in the OARDC dairy herd and to determine if the number was increasing, a herd survey was made on four separate occasions between 1964 and 1969. In each survey, a blood sample was collected from each available adult animal and evaluated by the Bendixen key. In addition, available records were searched for histologically confirmed cases of malignant lymphoma.

Results

One case of malignant lymphoma occurred in 1961, another occurred in 1963, and the most recent case occurred in 1968. No lymphocyte counts were in the leukemic range. The ranges and Bendixen evaluations are given in Table 3.

TABLE 1.—Details of Inoculation of Cattle Used in the Malignant Lymphoma Transmission Trials.*

Source of Inoculum†	Type of Inoculum‡	Route of Inoculation**	Amount of Inoculum	Animal Number	Age Group
Original case	Cells	IV	10 ml.	1534	1-6 mo.
Original case	Cells	IV	10 ml.	1588	1-6 mo.
Original case	Cells	IV	10 ml.	1597	1-6 mo.
Original case	Cells	IV	10 ml.	1601	1-6 mo.
Original case	Filtrate	IV	10 ml.	1542	1-6 mo.
Original case	Filtrate	IV	10 ml.	1591	1-6 mo.
Original case	Filtrate	IV	10 ml.	1599	1-6 mo.
Original case	Filtrate	IV	10 ml.	1602	1-6 mo.
Original case	Filtrate	IV	10 ml.	22	4-15 days
Original case	Filtrate	IV	10 ml.	23	1-3 days
Possible transmission	Filtrate	IV	10 ml.	24	1-3 days
Possible transmission	Filtrate	IV	10 ml.	46	1-3 days
Possible transmission	Filtrate	IV	10 ml.	47	4-15 days
Above two combined	Filtrate	SC	5 ml.	29	8 mo. fetus
Above two combined	Filtrate	SC	5 ml.	30	6 mo. fetus
Above two combined	Filtrate	SC	5 ml.	1704	6 mo. fetus
Above two combined	Filtrate	SC	5 ml.	31	5 mo. fetus
64-6	Whole blood	IV	100 ml.	1647	4-15 days
66-222	Filtrate	Multiple	20 ml.	1737	4-15 days
	Filtrate	Multiple	20 ml.	1738	1-3 days
	Cells	Multiple	20 ml.	1736	1-3 days
	Cells	Multiple	20 ml.	25	1-6 mo.
	Cells	Multiple	20 ml.	1	1-6 mo.
	DNA-RNA	Multiple	20 ml.	1735	1-3 days
67-26	Cells	Multiple	20 ml.	27	1-3 days
67-4	Cells	Multiple	20 ml.	26	16-30 days
67-4	Cells	Multiple	20 ml.	1733	1-6 mo.
67-4	Cells	Multiple	20 ml.	1734	1-6 mo.
67-4	Cells	Multiple	20 ml.	1739	1-6 mo.
67-70	Cells	Multiple	20 ml.	1771	16-30 days
67-70	Cells	Multiple	20 ml.	28	1-6 mo.

*The first eight animals listed were those reported in reference 11. 1602 was the possible transmission. All other listings are previously unreported.

†The numbers refer to histologically confirmed cases of bovine malignant lymphoma.

‡Filtrate — a cell-free filtrate prepared as previously described (1).

Cells — a minced cell suspension.

Whole blood — fresh citrated leukemic blood obtained from a clinical case of lymphoma.

DNA-RNA — Deoxyribonucleic and ribonucleic acid extract made from tumor samples.

**IV — intravenous
SC — subcutaneous
multiple — 5 ml. intravenously
5 ml. subcutaneously
5 ml. intraperitoneally
5 ml. into and around the prescapular lymph nodes.

TABLE 2.—Bendixen Evaluation of Cattle Used in the Malignant Lymphoma Transmission Trials.*

Animal	Inoculum†	Age at Death (mo.)	Bendixen Evaluation			Remarks
			Positive	Suspect	Total	
Inoculated Animals						
22	Filtrate	40	0	0	40	
23	Filtrate	59	0	0	59	
24	Filtrate	40	0	0	40	
46	Filtrate	31	14	24	106	‡
47	Filtrate	69	4	5	124	**
29	Filtrate	37	0	0	37	
30	Filtrate	35	0	1	35	
1704	Filtrate	Aborted				
31	Filtrate	34	1	0	34	
1716	Filtrate	3 days				
1647	Whole blood	59	4	17	102	††
1737	Filtrate	37	0	7	37	
1738	Filtrate	36	0	2	36	
1736	Cells	34	1	14	34	
25	Cells	36	33	3	36	
1	Cells	43	0	8	43	
1735	DNA-RNA	39	28	0	39	
27	Cells	37	0	0	37	
26	Cells	38	0	3	38	
1733	Cells	36	3	15	36	
1734	Cells	37	0	6	37	
1739	Cells	38	0	0	38	
1771	Cells	37	1	1	37	
28	Cells	35	0	2	35	
Contact Controls						
1809		45	0	0	45	
1815		27	0	0	27	
1810		20	0	0	20	
148		33	0	0	33	
Calves of Contact Controls						
107		21	1	0	21	
109		21	1	1	21	
106		20	1	0	20	
Calves of Inoculated Animals						
110		20	1	1	20	
111		21	5	4	21	‡‡

*A Bendixen evaluation is not available on the eight animals reported in the original transmission trial (11). All other animals are included in this table. No evidence of malignant lymphoma was found at necropsy on any animal in this table. Blood samples were evaluated at monthly intervals unless otherwise noted. The total number of Bendixen evaluations equals the age of the animal at death (in months) unless otherwise noted.

†Details of inoculation are given in Table 1.

‡Cow 46 died at 31 months of age from malignant catarrhal fever.

**Cow 47 was euthanized at 69 months of age.

††Cow 1647 was euthanized at 59 months of age.

‡‡The dam of calf 111 was cow 24.

TABLE 3.—Bendixen Evaluations of Animals in the OARDC Dairy Herd.

Date	Bendixen Evaluation			Percent Positive	Percent Suspect	Lymphocyte Range of Positive Samples	Lymphocyte Mean of Positive Samples
	Positive	Suspect	Total				
1964-1965	13	23	164	8	14	7,598-19,734	11,983
1967	16	25	246	7	10	7,656-20,430	12,040
1968	19	29	230	8	13	7,331-23,691	12,903
1969	8	19	188	4	10	7,126-14,696	10,268

LABORATORY ANIMAL TRANSMISSION TRIALS

Live malignant lymphoma cells failed to produce tumors when transplanted into the anterior chamber of guinea pig or rabbit eyes, the check pouch of young adult hamsters, or the abdominal cavity of newborn rabbits or hamsters.

Materials and Methods

Malignant lymphomas were collected at slaughter, direct impressions made from a freshly cut surface were stained with Wright's stain, and samples were preserved in 10% neutral formalin. A tentative diagnosis was made from the Wright's stained impressions and a final diagnosis was made from hematoxylin or azure and eosin stained sections of the formalin fixed tissues.

As soon as the tentative diagnosis was established from the direct impression slides, tumor samples were minced with sterile scissors in mammalian ringers solution[1] and manually agitated to create a cell suspension. The resulting suspension was filtered through three layers of cheese cloth, concentrated by centrifugation (500 g. for 15 minutes), and counted with an electronic particle counter.[2] The trypan blue exclusion test was used as a means of estimating cell viability. Suspensions containing more than 15% stained (dead) cells were not used as inoculums.

The intraocular injections were made by anesthetizing the test animal with ether, scrubbing the upper eyelid with alcohol, and inserting a 20 ga. needle through the eyelid, through the sclera just posterior to the limbus, and into the anterior chamber between the iris and lens. Inserting the needle through the eyelid minimized the eye rotation which caused difficulty with attempts to inoculate through the sclera alone. When the point of the needle could be seen between the pupil and lens, some of the aqueous humor was aspirated and replaced with inoculum.

Cheek pouch inoculation of hamsters was accomplished by anesthetizing the animal with ether, placing it under a magnifying lens, and everting the cheek pouch with small rat-toothed forceps. The cheek pouch was replaced in normal position following inoculation.

[1]NaCl 0.83%, KCl 0.02%, CaCl 0.02%, pH 7.2.
[2]Coulter counter.

TABLE 4.—Laboratory Animal Inoculations Using Bovine Malignant Lymphoma Cells.

Animal	Age Group	Number Inoculated	Number of Cells in Inoculation	Route of Inoculation	Losses Before End of Observation Period	No. Remaining at End of Obervation Period	No. Developing Tumors
Guinea pig	Adult	6	$9x10^5$-$1.3x10^6$	intra-ocular	0	6	0
Rabbit	Adult	10	$1.7x10^6$	intra-ocular	1 panophthalmitis	9	0
Rabbit	Newborn	8	$9x10^6$	intra-peri-toneal	0	8	0
Rabbit	Newborn	11	$8.9x10^6$	intra-peri-toneal	3 cannibalism	8	0
Rabbit	Newborn	3	$6.3x10^7$	intra-peri-toneal	0	3	0
Rabbit	Newborn	2	saline controls	intra-peri-toneal	0	2	0
Hamster	Newborn	13	$1.8x10^6$	intra-peri-toneal	8 cannibalism	5	0
Hamster	Newborn	18	$3.4x10^6$	intra-peri-toneal	1 cannibalism	17	0
Hamster	Newborn	5	saline controls	intra-peri-toneal	0	5	0
Hamster	Newborn	16	$9x10^6$	intra-peri-toneal	16 cannibalism	0	0
Hamster	Adult	12	$5.6x10^7$	cheek pouch	3 salmonellosis	9	0
Hamster	Adult	2	$2.8x10^8$	intra-peri-toneal	1 salmonellosis	1	0
Hamster	Adult	1	saline control	intra-peri-toneal	0	1	0

Intraperitoneally inoculated newborn rabbits and hamsters were frequently cannibalized by their mother. This problem was minimized by dusting the mother, litter, and the operator's hands with highly perfumed baby powder.

Following inoculation, the guinea pigs were observed for 3 months, the rabbits were observed for 4 months, and the hamsters were observed for 6 months. They were killed and examined at the end of the observation period. Six guinea pigs and nine rabbits were successfully inoculated in the anterior chamber. Nine young adult hamsters were successfully inoculated in the cheek pouch and one was inoculuated intraperitoneally. Nineteen newborn rabbits and 22 newborn hamsters were successfully inoculated intraperitoneally. Prednisolone was given to the adult hamsters daily for 1 week preceding and 3 weeks following inoculation. Animals which were cannibalized or died of intercurrent diseases were not included in the above listing. The complete tabulation is given in Table 4.

Results

No evidence of tumor formation was found in any animal. Inoculation into the anterior chamber resulted in clouding of the cornea within 48 to 72 hours, followed by gradual clearing. One of 10 inoculated rabbits developed granulomatous panophthalmitis, but all other rabbits and guinea pigs returned to normal within 60 days. Four of the 14 adult hamsters treated with prednisolone died of septicemic salmonellosis. Twenty-five of 47 newborn inoculated hamsters and 3 of 22 newborn inoculated rabbits were cannibalized.

SYNCYTIAL VIRUS ISOLATION

Following the report of the isolation of a syncytial virus from lymphosarcomatous and apparently normal cattle (7) and the suggestion that the virus might be involved in the development of bovine malignant lymphoma, an attempt was made to find if the agent was present in the OARDC dairy herd.

Materials and Methods

Five calves were obtained from the OARDC dairy herd. The calves had nursed their dams after birth and had subsequently been given at least one meal of pooled colostrum from the same herd. They were then housed together in the veterinary science facilities.

A spleen biopsy was obtained from each calf and tissue cultures were prepared, as described in the original report (7). Growth medium was changed twice a week and the cells were transferred once a week. The cells were inspected microscopically twice a week for evidence of syncytial formation.

A single hysterectomy-derived germfree calf served as a control. A spleen biopsy was obtained under germfree conditions and tissue cultures were made and maintained, as described above. This calf was subsequently inoculated intravenously with tissue culture spleen cells from one of the five conventional calves. The inoculum was a pooled suspension of cells from three 4-ounce prescription bottles. Each of the three bottles contained numerous syncytia at the time the inoculum was prepared.

Results

Syncytia formed in the spleen cell cultures from each of the five conventional calves. They were first found between the 2nd and 3rd week and became progressively more numerous with each cell transfer.

No syncytia were found in spleen cell cultures made from the initial spleen biopsy on the germfree calf. These cultures were discarded as negative after 6 weeks. Numerous syncytia formed in spleen cell cultures made from the biopsy obtained after the calf was inoculated with the cell suspension made from syncytia-containing cultures. The biopsy was made approximately 3 weeks following inoculation. The syncytia appeared approximately 2 weeks after the spleen cell cultures were made.

A BUFFY COAT STIMULATING EFFECT IN SERUM FROM CATTLE WITH PERSISTENT LYMPHOCYTOSIS

Introduction

An 8-year-old Holstein-Friesian cow in the OARDC dairy herd had lymphocytosis of 30 months duration, a history of gradual weight loss, partial anorexia, and decreased milk production. Malignant lymphoma was suspected because of palpable nodules in the region of the prescapular lymph nodes. A clinical study was undertaken.

As part of the study, buffy coat cultures were made. Washed buffy coat cells were suspended in bovine serum to a concentration of 2×10^7 cells per ml. Seven 4-ounce prescription bottles and six Leighton tubes were inoculated with cells suspended in homologous serum and an equal number were inoculated with cells in bovine serum which had been collected at slaughter. The leukocyte count of the slaughtered animal was unknown.

Approximately 4 months after the enlarged prescapular nodes were first noticed, the appetite, milk production, and weight began to increase and the palpable nodes began to recede. In the meantime, trypanosomes were detected in four of the seven prescription bottles and one of the six Leighton tubes containing buffy coat cells in heterologous serum. It was also noted that the cells grown in homologous serum were more abundant, larger, and more pleo-

morphic than those in the non-trypanosome infected cultures grown in heterologous serum.

At this point, a study was initiated to find if there was a buffy coat stimulating effect in serum from cattle with persistent lymphocytosis. The results are given below. A second study of the association between trypanosomes and lymphoctosis was also initiated and is reported in a later section.

Materials and Methods

The 8-year-old Holstein cow was used as a source of *lymphocytosis* serum. A 5-month-old Hereford with a consistently normal hemogram was used as a source of normal serum. Twenty-two cultures of buffy coat cells from the normal serum donor were grown in *lymphocytosis* serum and 21 were grown in normal serum.

Aliquots of *lymphocytosis* serum were filtered (0.45 micron Millipore filter[3]) or inactivated (56° C. for 60 minutes) and inoculated with buffy coat cells to determine the effects of these two procedures.

Five additional cows with persistent lymphocytosis and six cows with consistently normal hemograms were available. Leukocytes from each of these animals were cultured in homologous serum and the resulting growths were compared.

The details of cultural procedures have been reported (2).

Results

Growth of buffy coat cells was stimulated by serum from the 8-year-old Holstein with lymphocytosis. The stimulating effect was associated with the serum and not with the cells. The stimulating effect was not removed by filtration or heat inactivation. The buffy coat growth obtained in cultures from the five additional cows with persistent lymphocytosis was moderate to abundant, while the growth obtained in cultures from the six cows with consistently normal hemograms was very sparse to poor in five cows and moderate in one.

Details of evaluation have been reported (2).

TRYPANOSOMA THEILERI

Introduction

Following the isolation of *T. theileri* in buffy coat cultures from a cow with persistent lymphocytosis, a preliminary study was made to find if the association between trypanosome infection and lymphocytosis was common and whether trypanosome infection could cause lymphocytosis. Two splenectomized calves were inoculated intravenously with trypanosomes and both subsequently developed lymphocytosis.

A control calf, splenectomized to determine the effects of the surgery done, had no significant change in lymphocyte numbers. At this time, hemograms had been made at monthly intervals in 24 cattle. Six of these cattle had at least one hemogram with a lymphocytosis in the Bendixen positive range and one of them had persistent lymphocytosis. A blood sample from each animal was cultured for trypanosomes and they were isolated from three of the six animals with a history of lymphocytosis and one of the 18 animals with no history of lymphocytosis. Details of this trial have been reported (3).

It was believed that *T. theileri* might be involved in the development of bovine malignant lymphoma for the following reasons:

- *T. theileri* is a cosmopolitan parasite which has been reported from every country which cattle inhabit (6), and therefore it could be present wherever lymphoma occurred.
- *T. theileri* has been cultured from bovine buffy coat cells and could be expected to develop within neoplastic buffy coat cells.
- *T. theileri* might serve as a vector to carry a virus from infected buffy coat cells of one animal to noninfected buffy coat cells of another.
- By entering cells with latent infection, *T. theileri* might serve as a *trigger* for neoplasia.

If not directly involved in the pathogenesis, the presence of *T. theileri* as a concurrent infection which produced lymphocytosis in the Bendixen positive range could complicate the clinical diagnosis of lymphoma.

In order to investigate any of these possibilities, it was necessary to develop more basic information about the infection. The investigation therefore had the following goals:

1. Evaluate various methods of blood culture as a means of diagnosis.
2. Investigate other diagnostic methods.
3. Study the naturally occurring and artificially induced infection.

EVALUATION OF BLOOD CULTURE METHOD

One of the media first described by Splitter (10) was used as a routine isolation medium and a standard for comparison. Its composition is shown in Table 5. It was stored at —25° C. in 4 ml.

TABLE 5.—Composition of Splitter's Medium.

Calf serum	0.5 ml.
Veal infusion broth medium	1.75 ml.
Tissue culture medium (NCTC 109)	1.75 ml.
Heparin (1000 units/ml.)	0.01 ml.
Procaine penicillin G	200 units/ml.
Dihydrostreptomycin sulfate	250 mg./ml.
Inoculum (whole blood)	1.0 ml.

[3]Millipore Filter Corp., Bedford, Mass.

amounts in 15 x 150 mm. screw-cap vials. One ml. of blood was placed in a freshly thawed tube of medium as a standard inoculum. Inoculated tubes were incubated at 37° C. and samples were routinely examined for trypanosomes by bright field microscopy after 7 and 14 days. More frequent examinations were often made.

I. EFFECT OF ANTICOAGULANTS

Methods

A large blood sample was collected from a trypanosome-infected cow and 10 ml. aliquots were treated with commonly used anticoagulants, as shown in Table 6. Saline solution was added when necessary to bring all aliquots to a uniform 12 ml. volume. Each aliquot was then used to inoculate 10 tubes of medium which contained no anticoagulant. After incubation, the abundance of trypanosomes in a loopful of medium was estimated. In addition, blood from 32 cows was cultured in Splitter's medium made with and without heparin.

Results

Anticoagulants did not increase the number of isolations and may have inhibited trypanosome growth. The effects of various anticoagulants are shown in Table 7.

Five of the 32 cows surveyed had positive blood cultures in both media (with and without heparin) and the remaining 27 were negative. It is concluded that heparin does not influence trypanosome growth. Among other anticoagulants, sodium oxalate appears to reduce the growth.

II. EFFECT OF SERUM SOURCE IN MEDIUM

Methods

A 20 ml. sample of trypanosome-infected blood was collected and used (in 1 ml. amounts) to inoculate 10 tubes of Splitter's medium made with calf serum and 10 tubes made with lamb serum. The lamb serum was a pooled sample collected from several lambs selected at random. Blood from a group of 32 cows was also cultured in separate tubes of Splitter's medium made with each type of serum.

In a later trial, 10 tubes of inoculated medium made with lamb serum were compared with 10 tubes of medium made with chicken serum. The chicken serum was a pooled sample from several adult chickens selected at random.

TABLE 6.—Anticoagulants Added to Aliquots of Blood from a Cow Infected with Trypanosoma theileri.

Anticoagulant	Amount for 10 ml. Blood	Amount Saline
Sodium citrate	2 ml. of 3% solution	0
Sodium oxalate	2 ml. of 3% solution	0
Potassium oxalate	2 ml. of 3% solution	0
Oxalate mix*	1 ml.	1 ml.
E D T A (disodium)	1 ml. of 1% solution	1 ml.
Heparin	2 drops (1000 units/ml.)	2 ml.
Defibrinate	0	2 ml.
None	0	2 ml.

*Ammonium oxalate 1.2 gm.
Potassium oxalate 0.8 gm.
Distilled water 100.0 ml.

TABLE 7.—Effects of Anticoagulants on Trypanosome Growth.

Anticoagulant*	Growth in 7 days in Tube Number										Percent Positive
	1	2	3	4	5	6	7	8	9	10	
Sodium citrate	+++	+++	+++	+++	+++	+++	+	+	+	–	90
Sodium oxalate	+++	++	+	+	–	–	–	–	–	–	40
Potassium oxalate	++	++	++	+	+	+	+	+	+	–	90
Oxalate mix	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	100
EDTA	+++	+++	++	+	+	+	+	+	–	–	80
Defribrinate	+++	+++	+++	+++	+++	+++	+++	+++	–	–	80
None	+++	+++	+++	++	++	++	++	++	++	++	100

*Used as shown in Table 6.

Results

In the initial comparative culture, all 10 tubes made with lamb serum and all 10 tubes made with calf serum were positive for trypanosomes. They were generally more abundant in the medium made with lamb serum. In the cultures from the 32 cows, each medium supported trypanosome growth from the same seven infected cows after 7 days of incubation. By the 14th day, each medium detected infection in one cow that the other did not.

There was no growth in the 10 tubes of media made with chicken serum but all 10 tubes of the same medium made with lamb serum were positive for trypanosomes.

III. ALTERNATES FOR VEAL INFUSION BROTH

Methods

Splitter's medium was modified to support serial cultivation by adding bovine blood hemolysate and erythrocytes embedded in agar (8). This basic medium was then modified by using a variety of protein sources in place of veal infusion broth. Three tubes of basic medium (with veal infusion broth) and three tubes of each modification were inoculated with a 5-day-old culture of actively growing trypanosomes (1 ml./tube). After 7 days of incubation, growth in the basic medium was compared with growth in each modified medium.

Results

Media made with lactalbumin hydrolysate, brain heart infusion broth, and proteose peptone supported growth as well as veal infusion broth. The complete tabulation is given in Table 8.

TABLE 8.—Alternates for Veal Infusion Broth.

Enrichment	Percent Concentration	Growth
Veal infusion broth	2.5	4+
0.1% tryptophan + 0.5% lactose		±
Yeast extract	2.5	2+
Yeast extract	1.0	1+
Yeast autolysate	2.5	1+
Yeast autolysate	1.0	1+
Lactalbumin hydrolysate	2.5	5+
Lactalbumin hydrolysate	1.0	4+
Gelatin	2.5	—
Gelatin	1.0	—
Gelatin + 0.01% tryptophan	2.5	±
Gelatin + 0.01% tryptophan	1.0	±
Brain heart infusion	2.5	4+
Brain heart infusion	1.0	3+
Proteose peptone	2.5	4+
Proteose peptone	1.0	2+
Trypticase	2.5	±
Trypticase	1.0	±
Tryptone	2.5	2+
Tryptone	1.0	2+

LACTALBUMIN HYDROLYSATE MEDIUM

A simplified medium was made by adding 15% calf serum to 0.5% lactalbumin hydrolysate in Hanks' balanced salt solution. Phenol red was used as an indicator, the pH was adjusted to 7.4, and antibiotics were added as in Splitter's medium.

Blood from the same group of 32 cattle was cultured three times at monthly intervals in both lactalbumin hydrolysate and Splitter's medium.

Results

In the first month, each medium detected infection in two cows that the other did not and both detected trypanosomes in the same three infected cows. In the second month, each medium detected the same six infected cows. In the third month, each medium detected one infected cow that the other did not and both media detected the same four infected cows. It was concluded that the media were interchangeable for the purpose of primary isolation.

OTHER DIAGNOSTIC METHODS

Experimental Animal Inoculation

If *T. theileri* could be established in some animal smaller than a cow, it would greatly facilitate the development of diagnostic techniques. Therefore, an attempt was made to infect sheep, rabbits, guinea pigs, hamsters, and embryonated chicken eggs.

Methods

Six lambs between 1 and 3 months of age were each inoculated intravenously with 10 ml. of *T. theileri* culture in lactalbumin hydrolysate medium. Blood cultures were made twice from each lamb during the week before inoculation and twice weekly for 4 weeks after inoculation. Total and differential leukocyte counts were made on each sample.

Ten young adult rabbits were inoculated intravenously with 2 ml. of a similar culture. Blood cultures were made once before inoculation and once a week for 4 weeks after inoculation.

Five young adult guinea pigs and five young hamsters were inoculated intraperitoneally with 2 ml. of a similar culture. A blood culture from each hamster and guinea pig was made prior to inoculation and on the second and fourth week postinoculation. 0.5 ml. of blood was cultured each time from the guinea pigs and 0.25 ml. of blood was cultured each time from the hamsters.

Forty-six embryonated chicken eggs were inoculated with culture in lactalbumin hydrolysate medium. Ten additional eggs were inoculated with sterile medium. A standard volume of 0.2 ml. was used for each inoculation. Both allantoic cavity and yolk sac routes were used. Both 7- and 10-day-old embryos were used. The eggs were incubated under

TABLE 9.—Growth of Trypanosoma theileri **in Embryonated Chicken Eggs.**

No. of Eggs	Age at Inoculation*	Route	Results†		
			Days PI	Examined	Positive
26	10 days	Allantoic	4	8 died	5
			5	5 died	4
			6	13 killed	7
10	7 days	Allantoic	4	1 died	1
			5	1 died	1
			6	2 died	2
			13	6 killed	5
10	7 days	Yolk sac	1	1 died	0
			3	1 died	1
			4	1 died	1
			5	1 died	1
			13	6 killed	4
5‡	7 days	Allantoic controls	13	5 killed	0
5	7 days	Yolk sac controls	13	5 killed	0

*0.2 ml. inoculum was used in each case.

†Days postinoculation, number examined, number with trypanosomes.

‡The allantoic and yolk sac control eggs were inoculated with 0.2 ml. sterile medium.

standard conditions and examined for trypanosomes at various intervals after inoculation.

Results

All cultures from the lambs, rabbits, guinea pigs, and hamsters were negative for trypanosomes both before and after inoculation.

Trypanosomes were demonstrated in embryonated eggs from 3 days postinoculation until hatching. Details are given in Table 9.

DIRECT DEMONSTRATION

Methods

To determine the stain affinity of trypanosomes, known positive slides were prepared by placing a loopful of medium containing trypanosomes on 1 x 3-inch glass slides and air-drying them. After fixation in absolute alcohol for 2 minutes, representative slides were stained with Wright's, Giemsa's, Camco Quik Stain[4], methylene blue, Gram's, crystal violet, safranin, Gram's iodine, basic fuchsin, methenamine silver, hematoxylin and eosin, azure A and eosin.

Thin blood films had been made from 205 blood samples which had been positive for trypanosomes on culture. These were stained with either Wright's or Camco Quik Stain and examined for trypanosomes.

Buffy coats from 12 cattle known to have trypanosome infection were used to make thick blood films. These were stained with Camco Quik Stain and examined for trypanosomes. Prior to separating the buffy coat, each sample was cultured in lactalbumin hydrolysate medium to establish the presence of trypanosomes in the sample. Additional fresh unstained buffy coat preparations from these animals were examined microscopically, both as hanging drop preparations and as packed buffy coat columns in capillary tubes.

Lymph node and spleen specimens were collected from 13 cows with trypanosome infection. These were fixed in neutral formalin, sectioned at 6 microns following routine paraffin embedding, stained with azure and eosin, and examined microscopically for trypanosomes. All visceral tissues from one additional cow which had both malignant lymphoma and trypanosome infection were also examined.

Results

Methylene blue or related stains (Wright's, Giemsa's, azure) gave the most satisfactory results in staining slides made from the positive culture medium. Details of the rapid azure and eosin staining method have been published (1). No trypanosomes were found in the 205 thin blood films or the 12 thick buffy coat films made from samples known to contain trypanosomes. Trypanosomes were found in 1 of 12 packed buffy coat columns in capillary tubes. They were not found in the hanging drop preparations or in any of the tissue sections.

SEROLOGY

Source of Positive and Negative Serum

Serum samples were collected from four calves once or twice a week for 2 weeks before and 25 weeks after experimental infection with *T. theileri*. Total and differential leukocyte counts, packed cell volume

[4]Scientific Products, Evanston, Ill.

determinations, and trypanosome cultures were made at each collection. Trypanosomes were not isolated from any calf before experimental infection and were isolated from each calf following experimental infection.

Serum samples obtained before infection were considered as known negative samples. Serum samples obtained following experimental infection and re-isolation in blood culture were considered as known positive samples.

Method of Demonstrating Antibodies

A passive hemagglutination method using tanned formalinized bovine erythrocytes coated with antigen was used for antibody detection.

Bovine erythrocytes (RBC) were formalinized by the method of Csizmas (5), packed by centrifugation (750 g. for 15 minutes), and resuspended in a 1:5000 tannic acid solution in buffered saline (PBS)[5]. Following incubation at 37° C. for 30 minutes, the cells were washed once and resuspended in PBS to make a 10% suspension. Cells were coated with antigen by adding 0.2 ml. of packed RBC to 5 ml. of trypanosome homogenate and incubating 1 hour at 37° C. After incubation, the RBC were collected by centrifugation (750 g. for 15 minutes), washed three times, and resuspended to a 1% suspension in PBS containing 0.35% polyvinylpyrrolidinone. This suspension at pH 7.2 was used as antigen.

The trypanosome homogenate used to coat the cells was made by centrifuging trypanosomes in culture medium (8) at 10,000 r.p.m. for 10 minutes at 5° C. in a Spinco centrifuge. The sediment was washed once and resuspended to a 5% suspension in PBS and then homogenized in a Vertis Homogenizer Model 23 for 30 seconds at 45,000 r.p.m.

The test was conducted by making two-fold dilutions of serum in PBS containing 0.35% polyvinylpyrrolidinone. 0.2 ml. of each dilution was mixed with 0.2 ml. of the coated RBC suspension and incubated overnight at room temperature.

Results

Antibodies were not detected in any animal before experimental infection and were demonstrated in each animal after infection. The peak titer varied from a low of 1:16 to a high of 1:256. The initial reaction was demonstrated between 7 and 28 days postinoculation and the initial peak titer was reached between 23 and 65 days postinoculation. After the peak, the titer receded but three animals had a second peak. The second peak followed the first by 56 days, 104 days, and 151 days, respectively, in the three animals. One of the calves was negative for a period of 40 days between peaks. Titers of the four animals are shown in Figure 1.

The titers had no apparent correlation with trypanosome isolation or lymphocytosis.

[5]Buffered saline: NaCl, 36.00 g.; Na_2HPO_4, V.40 g.; KH_2PO_4, 2.15 g.; and Dist. H_2O, 1 l.

RELATIONSHIP OF T. THEILERI TO BOVINE LYMPHOCYTOSIS

Because preliminary work had demonstrated that lymphocytosis was more common in trypanosome-infected cattle than in negative cattle and because lymphocytosis had occurred in two splenectomized calves following artificial infection, a more extensive study of the association between lymphocytosis and trypanosome infection was started.

Materials and Methods

Naturally occurring *T. theileri* infection was studied in the OARDC dairy herd and in the experimental herd used for the lymphoma transmission trials. Blood samples were collected from the dairy herd during July and August in two successive years and from the experimental herd at monthly intervals. Total leukocyte and differential counts, packed cell volume determinations, and trypanosome cultures were made on each sample.

Infected animals in the dairy were ranged according to age in order to determine the prevalence of infection in various age groups. Monthly culture results from the experimental herd were tabulated to determine how consistently trypanosomes could be isolated from known infected animals. Trypanosome isolations were compared with Bendixen leukosis key reactions. The number of lymphocytes in trypanosome positive and negative blood samples taken at monthly intervals during a period of known trypanosome infection were tabulated.

The artificially-induced disease was studied in eight calves which were inoculated with infective blood. Blood samples were collected before and after experimental infection and the same determinations (listed above) were made. Prior to inoculation with infective blood, two of the eight calves were injected with blood containing dead trypanosomes. One of these calves was given a second inoculation of blood which contained dead trypanosomes 7 weeks after inoculation with infective blood.

Results

Natural infection by *T. theileri* was found in cattle of all age groups but it was rare in animals under 1 year of age. Infected animals were not consistently positive on consecutive blood cultures and there was considerable variation (in frequency of isolation) be-

tween infected individuals. Increasing the volume of blood cultured increased the number of isolations but did not eliminate periods of negative results.

Approximately 1 of every 5 trypanosome-infected samples had lymphocytosis in the Bendixen positive range while only 1 of every 20 trypanosome-negative samples was Bendixen positive. Trypanosome isolation was not a reflection of the number of mononuclear cells in the blood sample cultured.

Lymphocyte counts from seven of the eight experimentally infected calves rose above preinoculation levels and one remained essentially unchanged. The increase occurred between the second and fourth week postinoculation and ranged from 2000 to 6316, with a mean increase of 3549 lymphocytes per cu. mm. Lymphocyte numbers receded from the peak but never returned to preinoculation levels. Intravenous inoculation of blood containing dead trypanosomes did not cause lymphocytosis.

Details of this study have been reported (4).

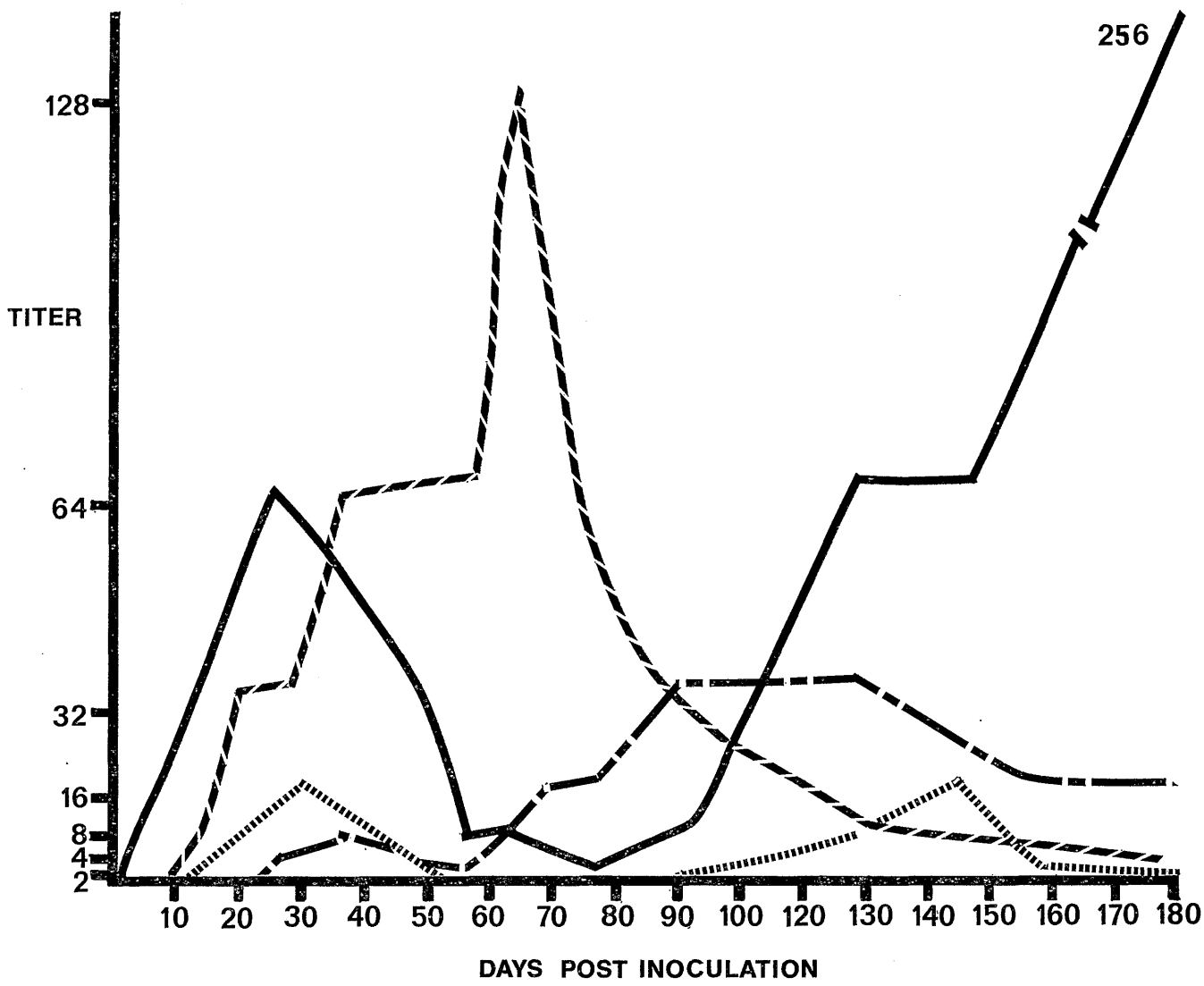

FIG. 1.—Passive hemagglutination titers of four calves with experimentally induced Trypanosoma theileri **infection.**

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